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Gut microbiota as a regulator of energy homeostasis and ectopic fat deposition: mechanisms and implications for metabolic disorders

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Purpose of review

To examine the role of gut microbiota in the regulation of host energy homeostasis and its role in the pathogenesis of obesity, diabetes and nonalcoholic fatty liver disease (NAFLD)

Recent findings

Experimental models highlight several mechanisms connecting gut microbiota to host energy metabolism: increased energy harvesting from the diet, regulation of appetite through gut peptide, secretion, regulation of tissue-free fatty acid composition and uptake, storage and oxidation, modulation of intestinal barrier by glucagon-like peptide-2 secretion, activation of innate immunity and hepatic fibrogenesis through the lipopolysaccharide (LPS)–toll-like receptor-4 axis.

Gut microbiota manipulation through antibiotics, prebiotics and probiotics yields encouraging results for the treatment of obesity, diabetes and NAFLD in animal models, but data in humans are currently scarce.

Summary

Gut microbiota manipulation yielded encouraging results for the treatment of different metabolic disorders in experimental models. However, changing intestinal microbiota may be more difficult in free-living individuals compared to standardized laboratory models, and its long-term consequences are unknown. To safely and effectively change human gut microflora, future research should highlight the complex hormonal, immunomodulatory and metabolic mechanisms underlying microbiota–host interactions in different tissues and candidate treatments should be evaluated in well designed trials with patient-oriented end-points.

Keywords

endotoxin, energy homeostasis, microbiota, NAFLD, obesity

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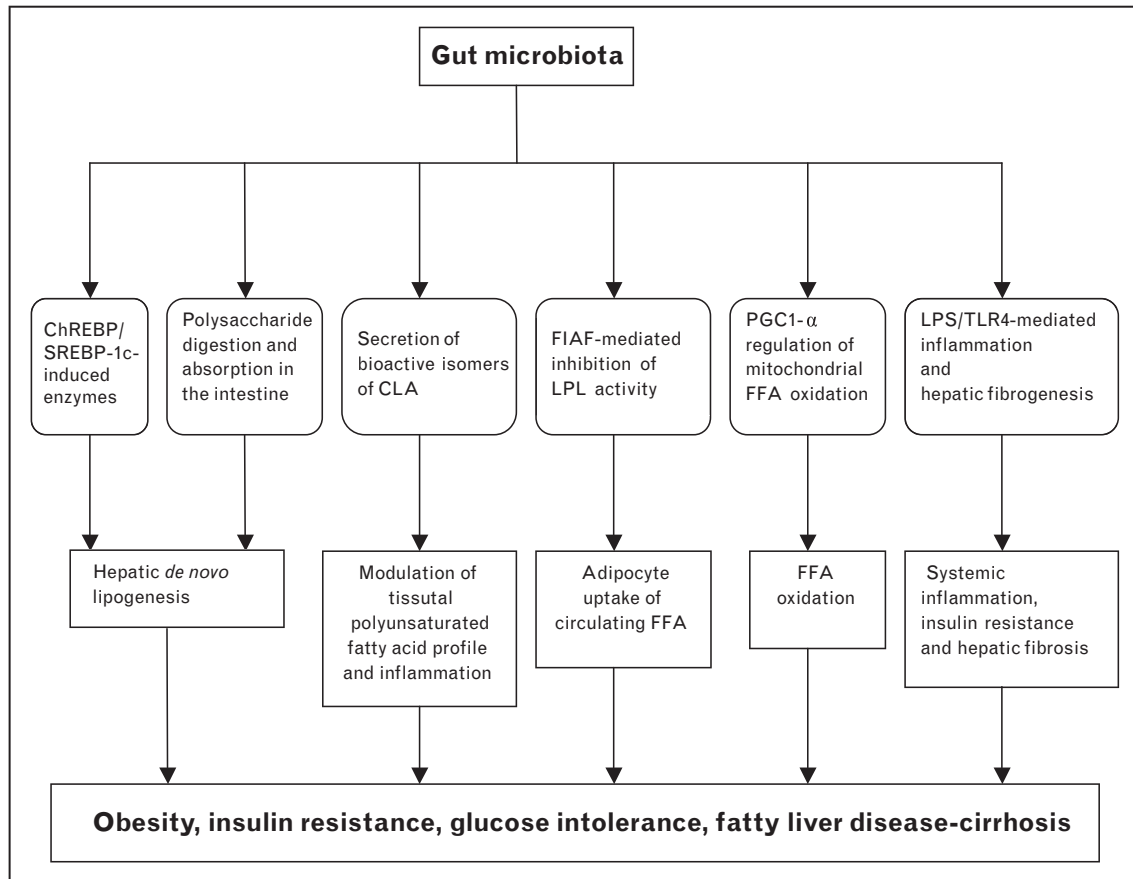
Introduction

Growing evidence demonstrates that the normal gut microbiome contributes to the development of diet-induced obesity. The human gut hosts 100 trillion microorganisms, encompassing hundreds of species. Colonic density of bacterial cells is estimated to be 10^{12} per ml, making the colon one of the most densely populated microbial habitats on Earth. The genome size of this microbial organ, collectively named ‘microbiome’, exceeds the size of the human nuclear genome by two orders of magnitude, providing the host with important biological functions. Recent research has highlighted some key aspects of the mammalian host–gut microbial relationship that could link gut microbiome to human obesity. We will review advances in understanding the role of gut microbiota in the pathogenesis of obesity, diabetes,

nonalcoholic fatty liver disease (NAFLD) and their potential therapeutic applications.

Role of gut microbiota in the regulation of fat storage

The involvement of gut microbiota in the regulation of host energy homeostasis has been first suggested by the pioneer experiments of J. Gordon’s group: they noticed that germ-free (i.e. raised in the absence of microorganisms) mice had 40% less total body fat than mice with a normal gut microbiota, even though the latter ate 30% less calories than did the germ-free animals [1]. If germ-free mice were ‘conventionalized’ with gut microbiota harvested from the cecum of a ‘normal’ mouse, they gained a 60% increase in body fat and insulin resistance within 2 weeks, despite a significant lower food intake.

Figure 1 Proposed mechanisms of the effects of gut microbiota on host metabolic and inflammatory processes

LPL, lipoprotein lipase; CLA, conjugated linoleic acid; ChREBP, carbohydrate-responsive element-binding protein; SREBP-1c, sterol-responsive element-binding protein-1c; FIAF, fasting-induced adipose factor; PGC1- α , peroxisomal proliferator-activated receptor coactivator-1 α ; LPS, lipopolysaccharide; TLR-4, toll-like receptor 4.

Subsequent research tried to elucidate the factors regulating gut microbiota composition and how it interacts with the host organism to influence the development of obesity and associated metabolic disorders. Mechanisms potentially underlying the weight gain under excessive caloric intake include an increase in the intestinal glucose absorption and energy extraction from nondigestible food component and concomitant higher glycemia and insulinemia. Glucose and insulin are known to promote hepatic de-novo lipogenesis through the expression of two key lipogenic enzymes, that is acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Consistently, a 2-week conventionalization of germ-free mice was associated with a two-fold increase in hepatic triglyceride content, accompanied by an increased hepatic mRNA expression of sterol-responsive element-binding protein (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP), two nuclear positive regulators of lipogenic enzymes [1,2 \bullet]. Furthermore, conventionalized mice had a higher monosaccharide uptake from the intestine to the portal blood, at least partly explained by the digestion of polysaccharides by microbial enzymes and by the higher

capillary density of the small intestine of conventionalized mice as compared to their germ-free counterparts. Lastly, the conventionalization also induced a systemic increase in lipoprotein lipase (LPL) activity, the enzyme catalyzing the release of free fatty acids (FFAs) and triacylglycerol from circulating triglyceride-rich lipoproteins to adipose tissue and muscle. The authors proposed that such an increase was the consequence of suppression of the fasting-induced adipose factor (FIAF) in the gut. FIAF inhibits the LPL activity (Fig. 1). The blunted FIAF expression in conventionalized germ-free mice might contribute to triacylglycerol accumulation in adipocytes and adipose tissue hypertrophy of conventionalized mice. This set of experiments demonstrated for the first time that an environmental factor such as gut microbiota may regulate energy storage.

Association between gut microbiota composition and obesity

The demonstration that obesity is accompanied by an altered microbiota composition in animals and humans

came subsequently from the same group [3,4]: upon characterization of over 5000 gut bacterial 16S RNA gene sequences, they found that genetically obese *ob/ob* mice had a 50% reduction in abundance of Bacteroidetes and a proportional increase in Firmicutes compared to their lean counterparts. In a parallel way, obese people were shown to have lower Bacteroidetes and more Firmicutes in their distal gut than did lean control individuals, alterations that were abolished after 52 weeks of diet-induced weight loss. To definitively assess if such different gut bacterial composition regulates body fat content, Turnbaugh *et al.* [5] transplanted cecal microbiota from lean and *ob/ob* mice to germ-free wild-type animals: after 2 weeks, rodents hosting the microbiota from obese mice increased their fat mass, and extracted more calories from food than the lean mice hosting the gut microbiota from lean mouse donors. Metagenomic analysis of the high-fat fed gut microbiome showed an increase in glycoside hydrolases, capable of breaking down otherwise indigestible alimentary polysaccharides, in transport proteins and enzymes involved in import and fermentation of simple sugars and host glycans, which can be utilized by the host for hepatic lipogenesis. As a consequence, hosts have an increased capacity to harvest energy from their diet.

Dietary fat determines the composition of the gut microbiome independently of obesity

To address whether the differences in gut microbiota composition between high fat-fed obese phenotype and lean phenotype [6] derive from the obese state or directly from the effects of different diet composition on bacterial populations, Hildebrandt *et al.* [7] employed the RELM β knockout mice, a phenotype that is resistant to high-fat-induced obesity. When RELM β knockout and RELM β wild-type mice were switched from a standard chow diet to a high-fat diet, the changes in the composition of the gut microbiome (expansion of the Firmicutes at the expense of the Bacteroidetes phylum) were similar between wild-type and knockout mice, indicating that effects of diet dominated. These findings were replicated by other groups ([6,9,36]) and indicate a high-fat diet, and not the obese state, can modulate microbiota composition by driving an increase in Firmicutes and a proportional decrease in Bacteroidetes.

Gut microbiota modulates the development of high-fat diet-induced obesity and insulin resistance

A further key experiment subsequently demonstrated that gut microbiota is an essential mediator of diet-induced metabolic disorders: Backhed *et al.* [8] fed germ-free or conventionalized mice a western diet (high fat/high carbohydrates). At the end of the experiment,

germ-free mice gained significantly less weight and fat mass than conventionalized mice, and were protected against the high-fat diet induced by glucose intolerance and insulin resistance. Differently from previous experiments, germ-free and conventionalized mice had a similar energy content in their feces, suggesting a more efficient energy harvesting from the high-fat diet which may not be the sole factor responsible for the fat mass gain of the conventionalized mice. The authors proposed two independent mechanisms, both resulting in increased FFA metabolism, at the basis of the resistance of germ-free mice to diet-induced obesity: elevated circulating levels of FIAF, which inhibits tissue LPL and increases expression of the peroxisomal proliferator-activated receptor coactivator (PGC)-1 α , a key regulator of enzymes involved in fatty acid oxidation; increased muscle and liver activity of the enzyme AMP-activated protein kinase (AMPK), which activates key enzymes of mitochondrial fatty acid oxidation, namely acetyl-CoA carboxylase and carnitinepalmitoyltransferase.

Thus, these data suggest that a bacterially related factor/mechanism other than energy harvesting may be responsible for the development of diet-induced obesity and diabetes.

Gut microbiota contributes to the low-grade inflammatory state of obesity

Gut microbiota has been recently linked to the low-grade chronic inflammatory grade which characterizes western diet-induced metabolic disorders. Specifically, the bacterial LPS, a cell-wall component of Gram-negative bacteria, could link gut microbiota to inflammation in obesity, diabetes, NAFLD, cardiovascular disease [9]. LPS is largely abundant in enteric Gram-negative flora and triggers the inflammatory process by binding to the complex of CD14 and the toll-like receptor-4 (TLR4) at the surface of innate immune cells [10]. More specifically, CD14 is a multifunctional receptor constituted by a phosphatidyl inositol phosphate anchored glycoprotein of 55 kDa expressed on the surface of monocytes, macrophages and neutrophils.

Cani *et al.* [6,11] demonstrated that after 2–4 weeks of high-fat feeding the mice exhibited a significant increase in circulating LPS levels, which they called ‘metabolic endotoxemia’, as LPS plasma concentrations were much lower than those commonly observed during septic shock. To assess the role of LPS as a trigger for the development of obesity and metabolic disorders, they reproduced the metabolic endotoxemia by chronically infusing mice with a very low dose of LPS to reach plasma LPS levels similar to those observed in the high-fat diet-fed mice [6]. After 4 weeks, LPS-infused animals developed the same phenotype as those on a high-fat diet,

namely, obesity, insulin resistance, diabetes, hepatic steatosis and insulin resistance, and adipose tissue macrophages infiltration. Finally, they challenged LPS receptor knockout (CD14KO) mice with a high-fat diet and, on a separate experiment, with a chronic low-dose LPS infusion. As expected, CD14KO mice were completely resistant to the development of insulin resistance and inflammation in the visceral and subcutaneous adipose depots, the liver and the muscle induced by both high-fat feeding or chronic low-dose LPS administration. Moreover, CD14KO mice were hypersensitive to insulin, even when they are fed a normal diet, suggesting that CD14 could be a modulator of insulin sensitivity in physiological conditions.

Taken together, these data support the concept that gut microbiota can play a key role in the pathogenesis of obesity-associated metabolic disorders.

Gut microbiota in the pathogenesis of nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease is considered the hepatic manifestation of metabolic syndrome and obesity. It encompasses a spectrum ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) – the latter potentially evolving to advanced fibrosis, cirrhosis and end-stage liver disease [12[•]]. Therefore, most of the current research in the field focuses on mechanisms leading to hepatic inflammation and fibrogenesis.

Different lines of evidence suggest gut bacteria may contribute to the pathogenesis of NAFLD. Plasma endotoxin levels are significantly higher in patients with NAFLD of different histological severity, from simple steatosis to NASH and fibrosis, and are associated with small intestinal bacterial overgrowth, increased intestinal permeability and with an induction of the endotoxin receptor TLR4 in the liver [13[•]–15[•],16]. Circulating levels of lipopolysaccharide-binding protein (LBP) have been found to be increased in patients with NAFLD and to a higher extent in patients with the progressive form of NAFLD, that is NASH, closely correlating with the increased hepatic expression of TNF- α [17]. The role of LPS-TLR4 axis in the pathogenesis of NASH was further substantiated by the observation that the functional deletion of TLR4 axis protected methionine-choline-deficient (MCD) diet-fed mice from the development of NASH [18].

The pathogenesis of increased intestinal permeability associated with bacterial overgrowth is not completely understood. Animal and human models of NASH suggest that bacterial metabolism of pyruvate, which is produced during the breakdown of carbohydrates, generates metabolites, including acetaldehyde and ethanol, toxic for the intestinal epithelium, leading to a disruption of the tight

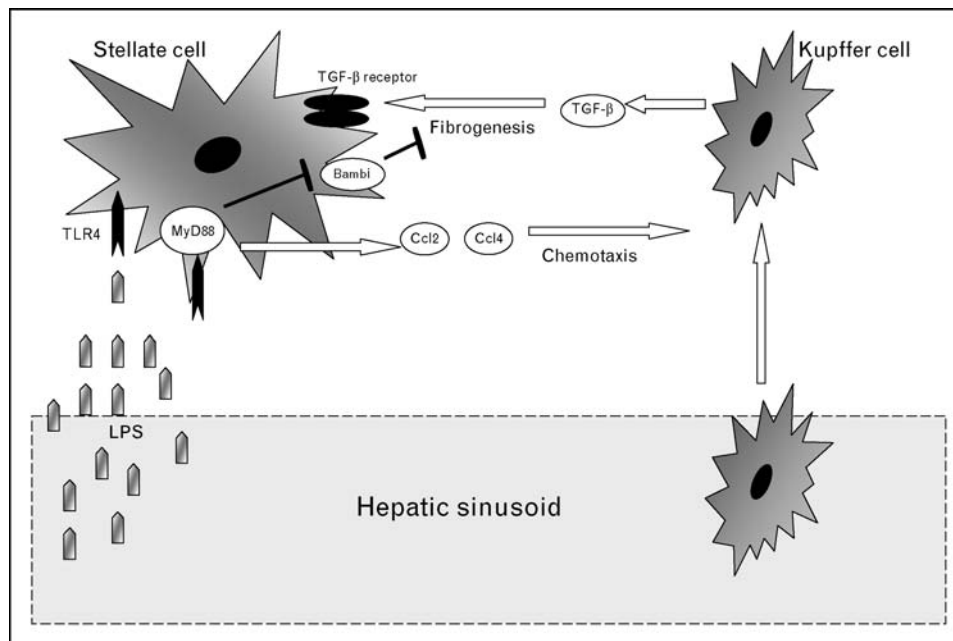
junctions [19,20]. Consistent with these findings, an excessive dietary fructose intake has been recently associated with the development of NAFLD in epidemiological studies, even in the absence of obesity, diabetes or other traditional risk factors [21[•],22]. To examine the interaction between fructose intake and LPS-TLR4-mediated hepatic steatosis, Spruss *et al.* [23^{••}] fed TLR4-mutant mice and wild-type mice with fructose or plain water for 8 weeks. Chronic fructose intake caused a significant increase in hepatic steatosis, lipoperoxidation and insulin resistance, coupled with a 22-fold increased hepatic expression of TNF- α and a 27-fold increase in portal endotoxin levels, in wild-type animals in comparison to water controls. All these alterations, except increased portal endotoxin levels, were significantly decreased in fructose-fed TLR4-mutant mice, suggesting LPS-TLR4 axis mediates the deleterious effects of excessive fructose intake on the liver. In line with this observation, the treatment with intestinal nonabsorbable antibiotics significantly reduced portal endotoxin levels, hepatic TNF- α expression, steatosis and liver injury in fructose-induced NAFLD animal models [24].

Apart from hepatic steatosis and inflammation, and most relevant for the hepatic complications of NAFLD, gut-derived LPS has also been directly connected to hepatic fibrogenesis via TLR4-mediated activation of hepatic stellate cells [25]. By challenging TLR4-knockout (TLR4-KO) mice with LPS, Seki *et al.* [25] showed that LPS directly activates hepatic stellate cells via a TLR4-dependent pathway. LPS enhances stellate cell activation by transforming growth factor (TGF)- β through down-regulation of the membrane receptor Bambi, a TGF- β pseudoreceptor with negative regulatory function. Activated hepatic stellate secrete chemotactic agents, including Ccl2 and Ccl4, to recruit Kupffer cells, which in turn secrete profibrogenic TGF- β , thus perpetuating the cycle (Fig. 2). Consistent with this model, the treatment with the probiotic VSL#3 led to upregulation of Bambi and ameliorated liver fibrosis in the methionine-choline-deficient (MCD) diet-induced mouse model of NASH [26^{••}].

Collectively, these experiments suggest that gut microbiota manipulation can help counteract the impact of unbalanced diets on the liver and may usefully add to other therapeutic options for NAFLD.

Gut microbiota as a modulator of cellular fatty acid membrane composition

An emerging mechanism whereby gut flora manipulation can affect host metabolism and fat storage is the modulation of fatty acid composition of host cellular membranes: different gut bacteria species produce bioactive isomers of conjugated linoleic acid (CLA) which exert a

Figure 2 Interaction between lipopolysaccharide, hepatic stellate cells and Kupffer cells in the liver

Toll-like receptor-4 (TLR-4) activation by lipopolysaccharide (LPS) downregulates, through the adaptor molecule MyD88, the membrane receptor Bambi, a pseudoreceptor for TGF- β with negative regulatory function. The removal of this inhibitory pathway leads to stellate cell activation and secretion of chemotactic factor Ccl2 and Ccl4 that recruit circulating macrophages to live to form Kupffer cells. Kupffer cells, in turn, secrete TGF- β and further activate fibrogenetic stellate cells.

variety of beneficial biological activities, including inhibition of cell proliferation, antiatherosclerotic, antidiabetic, immunomodulatory action and have the ability to reduce body fat [27]. Lactobacilli and Bifidobacteria from the mammalian gut, in particular, have been shown to generate CLA, predominantly the c9,t11 isomer, from free linoleic acid [28]. Wall *et al.* [29**] found that supplementation of *Bifidobacterium breve* to different mammalian species altered the profile of polyunsaturated fatty acid composition, resulting in higher intestinal, hepatic and adipose tissue content of c9,t11 CLA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These changes were associated with a reduced expression of proinflammatory cytokines TNF- α , IL-6 and IFN- γ [29**].

Evidence connecting high-fat diet, gut microbiota and metabolic disorders in humans

That differences in the gut microbiota may precede the development of obesity has been recently shown [30*]. Kalliomaki *et al.* [30*] found that *Bifidobacterium* spp., affecting both the quantity and quality of the microbiota during the first year of life, was higher in number in children exhibiting a normal weight at 7 years than in children developing overweight. Conversely, the fecal content of *Staphylococcus aureus* was lower in children remaining lean than in children subsequently becoming obese. This study suggests that the gut microbiota profile

in infancy may impact the risk of subsequent obesity, although it does not take into account other confounding factors, such as altered nutrient intake, nor provides a pathogenetic basis for this association. Creely *et al.* [31] found that endotoxemia was two-fold higher in the BMI, sex, and age-matched type 2 diabetes patients than in nondiabetic individuals. Furthermore, fasting insulin levels significantly correlated with LPS concentration in the nondiabetic population, even after adjustment for sex, age, and BMI [31]. Interesting data suggest that high-fat feeding is associated with a higher endotoxemia in humans. In healthy individuals, a high-fat meal induces a rapid increase in plasma endotoxemia to concentrations that are sufficient to activate cultured human aortic endothelial cells through the release of soluble TNF- α from monocytes [32]. A similar metabolic endotoxemia was able to increase adipose TNF- α and IL-6 concentrations and insulin resistance in another group of healthy individuals [33]. These experimental data were corroborated by a cross-sectional study, in which energy intake, especially different types of fat intake, was independently associated with metabolic endotoxemia in 211 healthy men [34*].

Molecular mechanisms underlying intestinal endotoxin absorption

Despite the growing body of evidence connecting gut-derived LPS to systemic inflammation and metabolic

disorders, little is known about mechanisms regulating intestinal LPS absorption. The observation that a high-fat diet increases plasma endotoxin levels 2–3-fold higher than high-carbohydrate diet suggests intestinal fat absorption and secretion may have a predominant role in LPS entry into the blood [6].

Using both animal models and cultured enterocytes, Ghoshal *et al.* [35^{••}] demonstrated that endotoxin is secreted into the circulation along with the formation and secretion of chylomicrons. Intragastric lavage with triolein (which forms chylomicrons) increased plasma endotoxin, whereas lavage with tributyrin (whose fatty acids enter the circulation without chylomicron formation) did not. Consistently, polarized CaCo-2 cells secrete endocytosed endotoxin when incubated with oleate, which forms chylomicrons, but not when incubated with butyrate, which does not. Importantly, inhibiting chylomicron formation blocked the effect of oleate. These findings suggest endotoxin is transported into the circulation in conjunction with chylomicron formation and secretion, not just translocated due to a breakdown of the intestinal barrier, as previously thought. If confirmed, these data raise the issue whether inhibiting chylomicron secretion may be effective for treating metabolic disorders even in the absence of overt hyperlipidemia.

Recent experimental data suggest gut microbiota may interact with the host at least in part through glucagon-like peptide-2 (GLP-2), a 33-amino acid peptide co-secreted with GLP-1 from enteroendocrine L cells in response to carbohydrate and fat ingestion, which has well known intestinotrophic properties.

Cani *et al.* [36^{••}] assessed changes in the gut microbiota, intestinal permeability and epithelial tight-junction proteins ZO-1 and Occludin, hepatic and systemic inflammation in genetically obese *ob/ob* mice following prebiotic or carbohydrate treatment. Prebiotic treated mice exhibited a lower plasma LPS and cytokines, and a decreased systemic and hepatic inflammation and oxidative stress, coupled with a lower intestinal permeability and maintained tight-junction integrity compared to controls, as expected. These beneficial effects were associated with an increased gut GLP-2 production, were abolished by the pretreatment with a GLP-2 antagonist and were mimicked by the administration of a GLP-2 agonist, thus suggesting GLP-2 may mediate many benefits of prebiotic treatment. However, to complicate this scenario, GLP-2 has been shown to increase intestinal lipid absorption and chylomicron production via CD36 activation, thereby potentially counteracting the beneficial effects observed with a carbohydrate diet [37]. The net effect of GLP-2 treatment on metabolic and inflammatory parameters under a high-fat intake, as well

as the mechanism(s) through which GLP-2 modulates intestinal enterocyte lipid and LPS absorption and secretion, remain poorly understood.

Treatment: animal models

Since obesity and high-fat intake are associated with a shift in the gut microbiota profile, with a relative reduction in *Bifidobacterium* spp. and *E. rectale*/Cl. *Coccoides*, proposed treatments aim at manipulating enteric flora by using intestinally focused antibiotics, probiotics (live bacteria given in oral quantities that allow colonization of the colon) or prebiotics (nondigestible oligosaccharides like inulin and oligofructose that are fermented by colonic microbiota and enhance the growth of beneficial commensal organisms like *Bifidobacterium* and *Lactobacillus* species). Importantly, *Bifidobacterium* spp. have been shown to reduce intestinal endotoxin levels and improve mucosal barrier function in rodents [38,39].

Cani *et al.* [11^{••}] treated *ob/ob* and high-fat fed mice with ampicillin and neomycin for 4 weeks. Antibiotic treatment dramatically changed the gut microbiota; reduced the *Lactobacillus* spp., *Bifidobacterium* spp.; and *Bacteroides-Prevotella* spp. All these features were associated with a strong decrease of metabolic endotoxemia, systemic inflammation, oxidative stress and macrophage infiltration in the visceral fat. Additionally, insulin resistance and glucose tolerance also significantly improved with antibiotics. To further demonstrate that the metabolic endotoxemia *per se* triggered the inflammatory state in these animals, the authors blocked the endogenous LPS action by administering an LPS quencher molecule, inactivating the circulating LPS, or by using a genetic model of obese mice lacking the LPS receptor CD14, the double knockout mice *ob/ob-CD14^{-/-}*. In both models, impairing the endogenous LPS action restored the phenotype observed during the antibiotic treatment. Other experiments using antibiotics or prebiotics obtained similar results [24,26^{••},36^{••},40–42].

Treatment: human data

Few well designed studies assessed the effects of probiotics/prebiotics on different metabolic end-points in humans.

Cani *et al.* [43^{••}] examined the effects of prebiotics on satiety and related gut-derived hormones following a test meal in healthy volunteers. They randomized 10 healthy adults to either 16 g prebiotics or 16 g dextrin maltose daily. After 2 weeks, prebiotic treatment increased gut microbiota fermentation, lowered appetite and improved postprandial plasma glucose responses. These effects were accompanied by an increase in plasma glucagon-like peptide 1 and peptide YY concentrations.

Nilsson *et al.* [44] assessed the effects of indigestible carbohydrates of an evening meal on glucose tolerance and related variables at subsequent standardized breakfast meal in 15 healthy individuals.

They found that the enrichment of indigestible carbohydrates of the evening meal improved glucose tolerance and adipokine profile at the subsequent breakfast. Such benefits correlated with the degree of colonic fermentation as assessed by breath hydrogen test.

Parnell and Reimer [45] examined the effects of oligofructose supplementation on body weight and satiety hormone concentrations in overweight and obese adults. They randomized 48 otherwise healthy overweight adults to 21 g oligofructose or placebo. After 12 weeks, oligofructose supplementation was associated with weight loss, reduced caloric intake and improved glucose tolerance. These changes were associated with reduced postprandial ghrelin and increased peptide YY responses

Finally, an open-label pilot study assessing the effects of probiotic VSL#3 in NAFLD was prematurely stopped because of significant increase in liver fat content after 4 months of treatment, an effect reversed after wash out of the drug [46].

Conclusion

Evidence is growing that the gut microbiota composition can modulate energy homeostasis and systemic inflammation and may thus contribute to the pathogenesis of different metabolic disorders.

Experimental models have highlighted several potential mechanisms at the basis of this association, that is energy harvest from the diet, regulation of fat storage through FIAF expression, regulation of lipogenesis and fatty acid oxidation, regulation of tissue polyunsaturated fatty acid composition, modulation of innate immune system activity, modulation of secretion of gut peptides (i.e. GLP-1, PYY) involved in hunger regulation. Gut microbiota manipulation also favorably affected different metabolic disorders in these models. Despite the growing experimental data, the evidence of effectiveness of these approaches in humans is still scarce for different reasons. First, manipulating human intestinal microbiota may be more difficult in human free-living individuals compared to standardized laboratory animal models. Second, the most effective type and dose of prebiotic to treat human disease are not yet established. Third, the hormonal, immunomodulatory and metabolic mechanisms underlying gut microbiota–host interactions in the intestine, liver, adipose tissue and inflammatory cells are only lately being unravelled and may differ between animal models and humans, among different organs/tissues and among

individuals with different metabolic milieu. Future studies need to highlight the molecular basis connecting gut microbiota to metabolic disorders and to address potential treatments in well designed trials with adequate clinical end-points.

Acknowledgements

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Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 88).

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